STUDIES OF HEMOLYTIC STAPHYLOCOCCI

HEMOLYTIC ACTIVITY—BIOCHEMICAL REACTIONS—SEROLOGIC REACTIONS

LOUIS A. JULIANELLE

Reprinted from THE JOURNAL OF INFECTIOUS DISEASES
Vol. 31, No. 3, September, 1922, pp. 256-284

STUDIES IN HEMOLYTIC STAPHYLOCOCCI

HEMOLYTIC ACTIVITY—BIOCHEMICAL REACTIONS—SEROLOGIC REACTIONS

A Thesis Submitted to the Graduate School of the University of Pennsylvania in Partial Requirement for the Degree for the Doctor of Philosophy.

R. UNIV.
BIBLIOTHEEK
LEIDEN

LOUIS A. JULIANELLE

Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41547328_0040

STUDIES OF HEMOLYTIC STAPHYLOCOCCI

HEMOLYTIC ACTIVITY—BIOCHEMICAL REACTIONS—SEROLOGIC REACTIONS

LOUIS A. JULIANELLE

From the Bacteriological Laboratories of the School of Hygiene, University of Pennsylvania and the Philadelphia General Hospital, Philadelphia

I. STUDY OF HEMOLYTIC ACTIVITY

That staphylococci lake blood was brought out in 1900, when Kraus [1] noticed the hemolytic effect of staphylococci on bloodplates. The following year Neisser and Wechsberg [2] demonstrated a hemolytic substance in filtrates of broth cultures. They found that in alkaline beef broth, this hemolytic substance began to appear on the fourth day and reached a maximum between the eighth and fourteenth day. In a general way they showed that aureus and virulent strains produced greater quantities of hemolysins than did either the albus or avirulent strains. Van durme [3] found that the hemolytic power was generally greatest in cultures freshly isolated from pathologic conditions, and was generally absent in cultures from dust and from the normal mouth. Todd,[4] working with B. megatherium and Kraus [5] working with staphylococcus showed that this action takes place in vivo as well as in vitro.

PRODUCTION OF HEMOLYSIN

It had been observed that in a general way staphylococci would show hemolysis to a greater or less extent on blood-agar plates within 24 hours. In addition, the hemolysis was not typical of an exogenous hemolysin, as is typical of Streptococcus hemolyticus; but rather resembled an exogenous product of metabolism, as in the case of B. coli, where the hemolysis diffuses haphazardly through the medium.

The first experiment was made to determine what analogy there was in chronicity in the production of hemolysins on blood plates and in broth. It might be stated here that all the work on hemolytic activity was obtained with 4 cultures representative of all the strains studied. Two were known hemolytic, and 2 were originally isolated as nonhemolytic. Twenty-four hour cultures were seeded into 10% horse (inactivated) serum broth in Erlenmeyer flasks and incubated at 37 C. for 24 hours. At the end of each 24-hour period, 5 c c of the culture were removed

Received for publication, May 22, 1922.

1 Wien. Klin. Wchnschr., 1900, 13, p. 49.
2 Ztschr. f. Hyg. u. Infektionskr., 1901, 36, p. 299.
3 Hyg. Rundschau, 1903, 13, p. 66.
4 Trans. London Path. Soc., 1902, 53, p. 196.
5 Wien. klin. Wchnschr., 1902, 15, p. 382.

aseptically and centrifuged at high speed for 5 minutes. One c c of the clear supernatant fluid was added to 1 c c of a washed 2.5% horse-blood suspension and incubated at 37 C. for 2 hours, at the end of which time the tubes were read for hemolysis. The concentration of blood attempted to approximate as closely as possible the conditions of the blood plate.

It was found that no estimable hemolysins were produced in broth cultures within 24 hours. In fact, as will be borne out later, no hemolysins were shown to be present until the sixth day. It may be that the discrepancy in time between plate and broth cultures is explainable on the grounds that in the former case the hemolysins are so concentrated around each colony as to assert themselves at a conspicuously earlier period; whereas in the latter case the hemolysins go into solution and become too dilute to have any effect on a suspension of blood cells.

The next experiment was planned to obtain the curve for the production of hemolysins. The technic employed was the same as in the preceding experiment, except for one detail. The cultures were seeded into tubes containing 10 c c of the serum broth, and at the end of each day one tube was removed from the incubator and used for the tests. Care was taken to keep the volume of the tubes constant by adding sterile salt solution to repair any loss by evaporation.

Table 1 shows that hemolysins begin to appear on the sixth day, reach a maximum at the ninth and tenth days, and disappear between the thirteenth and sixteenth days.

With the period of hemolysin production established, the logical sequence was to determine if possible the source or the cause of the production. It was assumed entirely theoretically that hemolysis is caused by one of the following or perhaps combination of factors:

1. Reaction: An increase or decrease in hydrogen-ion concentration sufficient to cause hemolysis.

2. Tonicity: An increase or decrease in the tonicity of the medium sufficient to cause crenation or laking of the blood corpuscles.

3. Hemotoxin: A hemolytic substance elaborated and secreted by the bacterial cell, causing hemolysis.

4. Proteolysis: The production by the bacterial cell of some substance for the utilization of the blood protein. Under this head would be included autolytic products also.

In order to establish experimentally which hypothesis was correct the following procedure was adopted: Coincidental with testing for the

presence of hemolysins, the hydrogen-ion concentration was read on the Clark and Lubs [6] scale; the amino acidity was titrated by the Sörensen [7] method; the proteose content was determined by the Vernon tests; [8] and numerical counts made at the end of each day, as long as was deemed necessary for the points at hand.

TABLE 1
PRODUCTION OF HEMOLYSINS

	Days															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Strain A1 (From Air)																
Hemolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amino acidity	54	56	56	56	56	54	58	58	50	50	50	50	4.6	38	36	36
Proteose content	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
H-ion concentration	7.9	7.9	8.0	8.1	8.2	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.4	8.4	8.4	8.4
Strain A5 (From Air)																
Hemolysis	0	0	0	0	0	0	±	+	+++	++	+1	+	+	+	±	0
Amino acidity	56	64	64	56	84	80	98	102	88	84	84	84	72	72	44	44
Proteose content	0.25	0.25	0.25	0.25	0.3	0.3	0.35	0.4	0.4	0.4	0.4	0.45	0.45	0.45	0.45	0.45
H-ion concentration	7.7	7.9	8.0	8.0	8.2	8.3	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4
Strain H2 (From Heart Blood)																
Hemolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amino acidity	54	56	56	56	56	56	54	52	46	44	44	44	38	36	34	34
Proteose content	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.3	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.3
H-ion concentration	7.7	7.9	8.0	8.2	8.2	8.2	8.2	8 2.	8.2	8.3	8.3	8.3	8.4	8.4	8.4	8.4
Strain T9 (From Throat)																
Hemolysis	0	0	0	0	0	0	±	+	++	+	+	+	±	0	0	0
Amino acidity	54	56	56	56	56	54	86	74	66	56	56	56	58	44	38	38
Proteose content	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.35	0.35	0.35	0.35	0.35
H-ion concentration	7.7	7.9	8.0	8.1	8.2	8.2	8.2	8.2	8.3	8.3	8.3	8.3	8.4	8.4	8.4	8.4

	Hemolysis	Amino Acidity	Proteose	H-ion
Control	0	38	0.25	7.5

Figures for proteose content represents amount of medium required to equal 1 c c of standard.

Figures for amino acidity represent c c of 20/N NaOH required to neutralize 100 c c of medium.

Plus signs indicate: +, 25% hemolysis; ++, 50%; +++, 75%.

Hours	Bacterial Counts Made With Production of Hemolysin Stains			
	A 1	A 5	H 2	T 9
0	40,000	72,000	50,000	73,000
24	100.000.000	180,000,000	450,000,000	300,000,000
48	830,000,000	900,000,000	850,000,000	1,000,000,000
72	1,210,000,000	6.300.000,000	8,900,000,000	9,200,000,000
96	7,000.000.000	1,000.000,000	2,500,000,000	2,000,000,000
120	460,000,000	300,000,000	3,200,000 000	800,000,000
144	150,000,000	350,000,000	800,000,000	600,000,000

1. An analysis of the results (table 1 and chart 1) shows several points: The ultimate reaction of all the cultures—hemolytic and non-

[6] Jour. Bacteriol., 1917, 2, p. 109.
[7] Biochem. Ztschr., 1908, 7, p. 45.
[8] Jour. Physiol., 1904, 30, p. 330.

hemolytic alike—is the same, P_H 8.4. If the hemolysis were the effect of reaction, all the cultures should show a like behavior on blood. But since the cultures do not show the same hemolytic activity, it is reasonable to exclude reaction as the cause of hemolysis. Incidentally, sterile salt solution, the reaction of which is adjusted to P_H 8.4, does not cause hemolysis.

Charts 1 and 2.—Showing counts, amounts of amino acids and hemolytic substances produced.

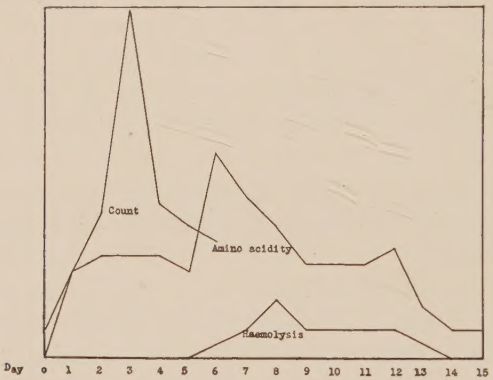

Fig. 1.—Strain T9.

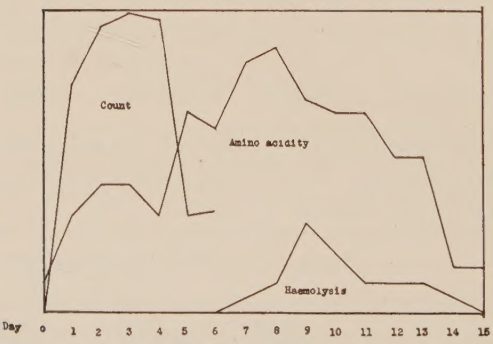

Fig. 2.—Strain A5.

2. No effort was made to determine the tonicity of the cultures. The impression was gathered from the work of Larson et al.[9] that bacteria of themselves do not change the surface tension of mediums, and in their study specific depressants were added when a drop in surface tension was desired.

[9] Larson, Cantwell, and Hartzell: Jour. Infect. Dis., 1919, 25, p. 41.

3. The figures for the numerical counts show that there is an increase in the number of staphylococci until the third day, when a maximum is reached. From then on there is a sharp decrease in numbers, indicating that growth of an active nature at least has come to a cessation. If the production of hemolysins and the numerical counts had shown a parallelism, it could have been reasonably assumed that the hemolysin were a true secretion product and a definite hemotoxin. Since they show no such parallelism, however, the hemolysin must be of some other nature.

4. The course of proteolysis or amine acidity runs a definitely parallel course to the curve of hemolysin production. The suggestion offered itself that if not directly associated, then some close relationship must exist between the two. Further study reveals the following concatenation of events: (1) the period of maximum growth occurs on the third and fourth day; (2) the maximum production of amino acidity occurs on the seventh and eighth days; (3) the maximum production of hemolysins occurs on the ninth and tenth days. Stated in another way, the growth period precedes the amino acidity period, which in turn precedes the hemolysin production period. It would seem from such an interrelated process that the production of hemolysins is a proteolytic process and perhaps even autolytic.

There is one other point of interest brought out by this experiment. Although there is an increase in amino acidity, there is no corresponding decrease in proteose content. This is probably due to the fact that the biuret test, used in determining the amount of proteose present, shows the presence of substances other than proteose; so that even if proteose were proteolyzed to form polypeptides, peptides and the higher amino acids the intensity of the color would still remain the same. One other point—it shows that the production of erepsin by staphylococci enables them to attack peptones and proteoses.

5. Following the suggestion offered in the foregoing experiment, the next step was to determine what rôle autolysis plays in hemolysis. For this purpose 24-hour cultures were inoculated in Erlenmeyer flasks (10% serum broth), and incubated at 37 C. for 5 days. This culture was then distributed in equal volumes into test tubes. To one series was added 0.25% phenol, to a second 10% HCC13; a third series was incubated at 45 C.; and a fourth was left untreated and incubated with the first and second series at 37 C. The object of this procedure was to determine whether after the maximum growth period was reached

and the cultures were inactivated by chemical or heat, with the enzymes still capable of activity, hemolytic substances were being produced. Each day tests were made for the presence of hemolysins. After the first day, guinea-pig serum and a living (24-hour) culture in 1 c c quantities were added to the 45 C. specimen. This was to supply complement, if it were needed, and any other vital substances necessary for hemolysis that a growing culture might possess. The results are appended in table 2, which shows: 1. No hemolysin was formed in cultures subjected to antiseptics or heat. 2. Complement does not appear necessary for hemolysis. 3. A living culture produces hemolysis per se, and is

TABLE 2

SHOWING EFFECT OF HEAT AND CHEMICAL AGENTS ON PRODUCTION OF HEMOLYTIC SUBSTANCE

	Days									
	1	2	3	4	5	6	7	8	9	10
Strain A5 (From Air)										
Phenol	—	—	—	—	—	—	—	—	—	—
HCCl3	—	—	—	—	—	—	—	—	—	—
45 C.	—	—	—	—	—	—	—	—	—	—
45 C. + complement	*	—	—	—	—	—	—	—	—	—
45 C. + 24 hour culture	*	+	+	+	+	+	+	+	*	*
Untreated	+	+	+	+	+	+	+	±	—	—
Phenol	—	—	—	—	—	—	—	—	—	—
HCCl3	—	—	—	—	—	—	—	—	—	—
45 C.	—	—	—	—	—	—	—	—	—	—
45 C. + complement	*	+	—	—	—	—	—	—	—	—
45 C. + 24 hour culture	*	+	+	+	+	+	+	+	*	*
Untreated	+	+	+	+	+	+	+	±	—	—
Phenol	—	—	—	—	—	—	—	—	—	—
HCCl3	—	—	—	—	—	—	—	—	—	—
Complement	*	—	—	—	—	—	—	—	—	—
Strain A5	*	+	+	+	+	+	+	+	*	*
Strain T9	*	+	+	+	+	+	+	+	*	*
Salt	—	—	—	—	—	—	—	—	—	—

* = no test conducted. Day 1 is 1st day under treatment, but 6th day of age of culture.

consequently worthless in such an examination. These results do not show that hemolysin is of an autolytic nature; neither do they show that it is not of an autolytic nature. The conclusion to be drawn is that in the case of heat, the hemolysin being thermolabile, is possibly dissipated; while in the case of the antiseptics, the hemolysin is so closely associated with the bacterial cell that destruction of the latter means lack of manifesation from the former. This falls somewhat in line with the work of Gordon on meningococci, showing that hemolysins are endocellular and are liberated on autolysis of the bacterial cells.

Effect on Hemolytic Activity of Successive Transplantation in Blood-Free Medium.—The object of the next experiment was to

determine whether a hemolytic strain of staphylococcus is always hemolytic. No definite references to the loss of this haemolytic manifestation could be found in the literature. Transplants were made daily into peptone broth, and at the end of each week blood-agar plates were streaked to show whether the cultures were still hemolytic. After the second week, since the plates were readily hemolyzed, the cultures were transplanted every other day, and after the first month every week. The reason for this change of procedure was the assumption that by daily transplantations the cultures were kept very active and that it would be more difficult, if possible, to suppress so vital a quality.

This experiment was continued for more than four months, and at the time of writing the cultures were still hemolytic. On some occasions there appeared to be retardation in hemolysins, and then the following week the cultures were as actively hemolytic as originally. Since the retardation was neither progressive nor continuous, it is reasonable to assume that it was probably due to differences in the blood used for the work. Normal horse blood, which was used for the blood-agar plates in this experiment, has been shown by Neisser and Wechsberg [2] to possess small quantities of antihemolysin. This normal quantity, however, may have been sufficient to delay nemolysis. It would seem, therefore, that hemolytic cultures tend to remain hemolytic.

Effect on Nonhemolytic Strains of Successive Transplantations in Blood Medium.—In this case the point at hand was to determine whether nonhemolytic cultures could be made hemolytic by adaptation to blood medium. If nonhemolytic cultures can be made to lake blood, it may be said that any strain of staphylococcus is hemolytic, adding provisionally that continual adaptation to a blood-free habitat ultimately suppresses its hemolytic activity and keeps it in abeyance; but readaptation to a blood-containing medium will restore the suppressed activity. Table 3 shows that after a period of 7 months, certain strains regained their hemolytic ability. It may be that this power was recovered at an earlier period, but tests were not definitely made until the stated lapse of time.

It should be added here, in view of a wealth of work in a hospital laboratory, that we think every strain of staphylococcus is definitely hemolytic. The strains will vary in degree of hemolysis, and in rapidity of hemolysis, but if sufficient time is given, all strains will show hemolysis. When the strains under study were isolated, a period of 6 days was given to determine hemolysis on blood plates, and it is now apparent

that the 6 days were not sufficient. Other cultures not included in this survey did show hemolysis after the arbitrarily chosen time, and attempts to collect nonhemolytic strains after 10 and in rarer cases 12 days, have failed. So that it seems by virtue of this evidence that the strains we originally labeled nonhemolytic were in reality hemolytic, and that their hemolytic character was very much suppressed. It would seem, therefore, that it can be definitely stated that cultures which did not show hemolysis within 6 days were able to give definite signs of hemolysis on blood plates within 24 hours and complete hemolysis within 48 hours.

Since the completion of this experiment every strain of staphylococcus isolated (whether a contaminant or a pathogen) was held for study. The number of days required to show beginning hemolysis was recorded. These results are tabulated in table 4. It will be seen that every strain shows hemolysis, but that the factor of time plays an

TABLE 3

DEVELOPMENT OF HEMOLYSIS BY NONHEMOLYTIC ? STRAINS

	April, 1921	November, 1921		April, 1921	November, 1921
A 1	No hemolysis	Hemolysis	P 5	No hemolysis	Hemolysis
A 2	Hemolysis	Hemolysis	S 2	Hemolysis	Hemolysis
A 3	Hemolysis	Hemolysis	T 1	No hemolysis	Hemolysis
A 5	Hemolysis	Hemolysis	T 2	Hemolysis	Hemolysis
F 1	No hemolysis	Hemolysis	T 3	Hemolysis	Hemolysis
H 2	No hemolysis	Hemolysis	T 5	Hemolysis	Hemolysis
P 1	Hemolysis	Hemolysis	T 6	Hemolysis	Hemolysis
P 2	Hemolysis	Hemolysis	T 8	No hemolysis	Hemolysis
P 3	Hemolysis	Hemolysis	T 9	Hemolysis	Hemolysis
P 4	Hemolysis	Hemolysis	X	No hemolysis	Hemolysis

important part. Thus it is seen that in a general way aureus strains show hemolysis earlier, and that virulent strains also show hemolysis earlier than the saprophytic; but the point is clear that white and aureus strains, saprophytic and parasitic alike, become hemolytic. In the case of nipples, for example: These are supposedly sterilized and sent to the laboratory to be tested for sterility so that it is logical to assume that any growth is apt to be contamination. Yet the 9 white strains are as rapidly hemolytic as the 18 orange strains isolated from pus.

Effect of Carbohydrates on Hemolysis.—It has been shown by Ruediger,[10] Lyall,[11] Davis,[12] Sekiguchi,[13] Stevens and Koser[14] and

[10] Ibid., 1906, 3, p. 663.
[11] Jour. Med. Res., 1914, 30, p. 515.
[12] Davis: Jour. Infect. Dis., 1917, 21, p. 308.
[13] Ibid., 1917, 21, p. 475.
[14] Jour. Exper. Med., 1919, 30, p. 539.

others, that carbohydrates prevent hemolysis by streptococcus, and it was problematic just what their effect on staphylococcus hemolysis would be. Two experiments were carried out to determine this point. In the one case, cultures were planted into 10% serum broth plus 1% dextrose. After 9 days tests were made for hemolysis and H-ion concentration read—to assure ourselves that an acidity would not interfere with the test. The results were: for 10% serum broth, dextrose 1%, strain A5 gave P_H 6.4, hemolysis and strain T9, P_H 7.6 and hemolysis.

Incidentally both these strains were streaked on lactose-blood-agar plates, and in both cases hemolysis was produced within 24 hours. In the second case, cultures were planted into peptone broth plus 1% dextrose. The test for hemolysis was positive after 24 hours, but the

TABLE 4
HEMOLYTIC ACTIVITY OF CONSECUTIVE CULTURES

Source	Pigment	No. of Cultures	Average Time for Hemolysis
Air	White	8	6-7 days
	Yellow	2	1-2 days
Sputum	White	1	3 days
	Aureus	2	1 day
Skin	White	4	4 days
Throat	White	4	1 day
	Yellow	1	1 day
Tonsil	White	1	1 day
	Aureus	1	1 day
Nipple	White	9	1-2 days
	Aureus	2	1 day
	Yellow	2	1 day
Feces	White	2	3 days
	Yellow	1	2 days
Pus	Yellow	3	2 days
	Orange	18	1-2 days
Necropsy	Aureus	3	1-2 days
	Yellow	1	1 day
Water	White	2	4 days
Contamination (source unknown	White	3	7 days

hemolysis was not typical, showing a browning similar to acid hematin formation. Consequently the P_H value was determined and found to be 4.4. The reaction was adjusted to neutrality and hemolysis no longer took place. Approaching the question from another tangent, sterile salt solution adjusted to a reaction of P_H 4.4 caused the same type of hemolysis.

It would seem from these experiments that carbohydrates do not influence hemolysis as produced by staphylococcus. Regarding the acidity produced in the peptone broth and not in the serum broth, it is easily conceivable that the buffer qualities of the serum in the latter obscure the acid formed by fermentation of dextrose.

Effect of Heat on Hemolysis.—Neisser and Wechsberg [2] found that heating the staphylococcus "hemolysin" for 20 minutes at 56 C. would completely inactivate it.

In determining the effect of heat on the hemolytic action, the supernatant fluids of centrifuged 9-day cultures were heated at 56 C. for 30 minutes, and it was found that the hemolysin of staphylococcus is a thermolabile substance, which can be destroyed by heating in this way.

DISCUSSION

Previous investigators of the hemolytic activity of staphylococcus were concerned with observations of the hemolytic activity per se. Aside from some speculations as to its relation to pigment, virulence and agglutination, no attempt was made to arrive at its causation. The point under study here was concentrated on the cause of the hemolytic activity, and the period of its development was only a coincidental observation, since this phase of it was already sufficiently elaborated by previous investigators.

Our results point to a process of proteolysis—perhaps associated with autolysis—as the cause of hemolysis. This is not a new conception —it has been shown to be the fundamental of meningococcus hemolysis and were experiments performed to establish the point of possibly B. proteus, B. coli, etc. Although we have been unable to demonstrate irrevocably that autolysis is the specific cause, it is very significant that the period of maximum growth first appears, then the period of maximum amino acidity, and, finally, the period of maximum hemolysis. Such a sequence of evidence can point only to autolysis.

It must be for this reason that we have been unable to suppress the hemolytic activity of our hemolytic strains. If hemolysis is due to so important a function as protein-splitting, the factor involved is too vital to be eradicated by continued growth in blood-free mediums. Conversely, it is no wonder that slowly hemolytic cultures will increase in rapidity of hemolysis by continued adaptation to an environment where protein ultilization becomes more pronounced.

Nor is it phenomenal that sugar should not inhibit hemolysis in such a case. Kendall and Walker's [15] conception that the presence of glucose has a protein sparing effect and consequently retards production of proteolytic enzymes can be accepted only provided the hydrogen-ion concentration of the medium increases within suitable limits. For as

[15] Jour. Infect. Dis., 1915, 17, p. 442.

Berman and Rettger [16] pointed out, in tests in which buffers are employed proteolytic enzymes appear as soon in sugar mediums as in plain broth. And in serum broth, the buffer qualities of serum cannot be denied.

II. RELATIONSHIP OF HEMOLYTIC ACTIVITY TO OTHER METABOLIC ACTIVITIES

This part of the investigation concerns itself with a study of the biochemical reactions of the staphylococci, particularly as possible relations to hemolysis. Although, as the evidence submitted will show, hemolysis appears to be a separate entity from the biochemical reactions pursued, some new points of interest have been added to the literature of the hemolytic staphylococci.

CHROMOGENESIS

Except in a general way, a distinction of the chromogenic varieties of the staphylococci is an insignificant one. The pigment produced by bacteria is influenced to a greater or less extent by the medium employed for its production, and can be greatly modified by selection or by previous environment. Loeffler's serum medium, for example, without affecting the inherent power of chromogenesis always accentuates the depth of color produced by staphylococci. Pigment will vary with the amount of oxygen, the amount of moisture available, and the age of the culture.

So Neisser and Lipstein [17] offer the hypothesis that white cocci were originally orange cocci which have lost their chromogenic power. Rodet and Courmont [18] published the observation of the transformation of a white staphylococcus to an aureus and subsequently to a white again. Lubinski [19] showed that the orange forms lost their pigment when grown anaerobically; in some cases the recovery was delayed and in other cases the loss was permanent. Kolle and Otto [20] stated that chromogenic cocci lose their chromogenesis by heating to 85 C., by prolonged cultivation on artificial mediums, and by repeated animal passage. Winslow and Rogers [21] showed that a temperature of 50-55 C. may cause a loss in chromogenesis.

Neisser and Wechsberg showed that strains of both Staphylococcus albus and Staphylococcus aureus would produce hemolysins. This was later corroborated by both Kutscher and Konrich [22] and Koch.[23] Noguchi [24] and Rosen-

[16] Jour. Bact., 1918, 3, p. 389.
[17] Handbuch. d. pathog. Mikroorganismen, 1914, 3, p. 105.
[18] Compt. rend. Acad. d. sc., 1890, 9, p. 186.
[19] Centralbl. f. Bakteriol., 1894, 16, p. 769.
[20] Ztschr. f. Hyg. u. Infektionskr., 1902, 41, p. 369.
[21] Jour. Infect. Dis., 1906, 3, p. 485.
[22] Zetschr. f. Hyg. u. Infektionskr., 1904, 48, p. 249.
[23] Ibid., 1907, 58, p. 287.
[24] Arch. f. klin. Chir., 1911, 96, p. 696.

bach[25] show a relation between virulence and pigmented cocci, while Passet[26] and Fisher and Levy[27] show that the lightly colored or colorless forms are most often associated with disease processes.

EXPERIMENTS

In determining chromogenesis the technic employed was that suggested by Winslow and Winslow.[28] Cultures were grown on agar slants at 20 C. for 2 weeks. A portion of the growth was spread over white roughened paper, with a platinum loop and allowed to dry in air. The hue and tint were matched against the colors of the frontispiece of their book (table 5).

TABLE 5

SOURCES AND CHROMOGENESIS OF THE STRAINS STUDIED

A 1—From the air	Lemon yellow I
A 2—From the air	Medium cadmium yellow IV
A 3—From the air	Cadmium orange III
A 5—From the air	Medium cadmium yellow IV
F 1—From feces	White
H 2—From heart's blood at necropsy	Medium cadmium yellow V
P 1—From pus from spine	Cadmium orange IV
P 2—From pus from carbuncle	Lemon yellow I
P 3—From pus from acne	Cadmium orange IV
P 4—From pus from extracted tonsil	Cadmium orange IV
P 5—From pus (unclassified)	Cadmium orange IV
S 2—From skin	White
T 1—From throat	Orange yellow III
T 2—From throat	Medium cadmium yellow V
T 3—From throat	Medium cadmium yellow VI
T 5—From throat	Lemon yellow II
T 6—From throat	Lemon yellow III
T 8—From throat	White
T 9—From throat	Orange yellow V
X—From blood culture (case furunculous)	Orange yellow III
C15—From throat	White
C16—From throat	White
C18—From throat	White
J 1—From pus	White
L 1—From pus	Cadmium orange IV

It will be seen at a glance that there is no relationship between pigment and hemolysis. The cultures are all hemolytic, and yet they vary from a white to a rich golden brown. This is scarcely surprising. The literature shows that pigment production may be varied, and while the hemolytic activity seems to be fixed, it could hardly be expected that the two functions would be related.

ACID PRODUCTION IN THE PEPTONE MEDIUM OF CLARK AND LUBS

Preparatory to the carbohydrate metabolism studies of staphylococci, this experiment was made to determine in a general way any relation-

[25] Dent. med. Wchnschr., 1884, 6, p. 31.
[26] Passet: Fortschr. d. Med., 1885, 33, p. 33.
[27] Dent. Ztschr. f. Chir., 1893, 36, p. 94.
[28] Systematic Relationships of the Coccocese, 1908.

ship between hemolysis and acid production. In view of the methyl red test of differentiation of B. coli and B. aerogenes by this medium, it seemed at the time that it might possess some value in this work. The peptone medium contained 0.5% K_2HPO_4, 0.5 peptone (Difco), and 0.5% dextrose, and was adjusted to P_H 7.4.

Table 6 shows the H-ion readings of the different cultures from time to time as specified. With the exception of A1, all strains reach an end-point of P_H 4.2-4.6 within 96 hours. Although there seem to be differences in the earlier readings, there is no line of demarcation between the acid production of the cultures. These differences are probably explainable on differences in numbers inoculated, periods of lag, etc.

TABLE 6

ACID PRODUCTION IN CLARK AND LUBS MEDIUM *

	8 Hours	12 Hours	16 Hours	20 Hours	24 Hours	48 Hours	72 Hours	96 Hours
A 1	7.6	7.6	7.4	7.0	6.9	6.9	6.9	6.9
A 2	6.4	4.6	4.6	4.6	4.6	4.4	4.4	4.4
A 3	6.2	4.6	4.4	4.4	4.4	4.4	4.4	4.4
A 5	4.6	4.4	4.2	4.2	4.2	4.2	4.2	4.2
F 1	6.0	5.8	5.8	5.0	4.6	4.6	4.6	4.6
H 2	5.0	4.6	4.4	4.4	4.4	4.4	4.4	4.4
P 1	6.1	4.9	4.6	4.6	4.6	4.6	4.6	4.6
P 2	6.0	4.6	4.6	4.6	4.6	4.6	4.6	4.6
P 3	6.8	4.6	4.6	4.6	4.6	4.6	4.6	4.6
P 4	5.0	5.0	4.8	4.6	4.6	4.6	4.6	4.6
P 5	6.6	5.0	4.6	4.4	4.4	4.4	4.4	4.4
S 2	7.6	5.8	5.0	5.0	4.6	4.6	4.6	4.6
T 1	5.0	5.0	4.8	4.8	4.6	4.6	4.6	4.6
T 2	5.4	4.8	4.8	4.6	4.6	4.6	4.6	4.6
T 3	5.0	4.9	4.9	4.9	4.6	4.4	4.4	4.4
T 5	5.0	4.8	4.6	4.4	4.4	4.4	4.4	4.4
T 6	7.6	6.6	6.0	5.4	4.6	4.6	4.6	4.6
T 8	5.5	5.0	5.0	4.8	4.8	4.8	4.6	4.6
T 9	7.4	5.6	5.0	5.0	4.6	4.6	4.6	4.6
X	6.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
Control	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2

* Figures represent values of H-ion concentration.

At this point, the question arose as to what determined the acid end-point of the cultures. To approach an answer, 2 experiments were planned: (1) Cultures were grown in the same medium with the reaction adjusted to P_H 4.4; (2) cultures which had already reached an acidity of P_H 4.4 were killed by heating at 56 C. for 30 minutes and inoculated with a 24-hour culture.

In both these cases, the H-ion concentration was increased to 4.2 and 4 after 24 hours. It might be of interest to quote here the work of Hall and Frazer [29] who found that staphylococci could reach a H-ion concentration of 2.6—an end-point which exhibited no relation to saprophytic or pathogenic forms.

[29] Abstract, Lancet, 1921, 18, p. 912.

CARBOHYDRATE METABOLISM

In view of the diagnostic importance of the fermentative reaction of the colon-typhoid group, it was deemed advisable to devote considerable attention to this subject. Very little previous work has been done on the ability of the staphylococci to ferment carbohydrate mediums. Of course, it is common knowledge that they attack the more familiar sugars with the production of acid, but no gas. Gordon,[30] in reporting a classification study of the white cocci, gave the fermentation reactions on lactose, maltose, glycerol and mannitol. Dudgeon[31] reported a comparative study of the aureus and albus cocci, studying among other things their acid production in 11 carbohydrate mediums; but none of his results were quantitative. Winslow and Winslow[28] studied glucose and lactose, and Kligler[32] glucose, lactose and sucrose. More recently Winslow and his co-workers[33] made a quantitative study of the acid produced in 9 different sugars. They found more than half the strains studied fermented glucose, maltose and sucrose; about half fermented lactose; 5 strains fermented salicin, 1 strain each fermented inulin and raffinose, and no strains fermented dulcitol and mannitol.

In our study, we have employed 17 carbohydrates in all-dextrose, galactose, levulose, sucrose, lactose, maltose, raffinose, arabinose, inulin, dextrin, salicin, adonitol, mannitol, sorbitol, dulcitol, glycerol and starch. Twenty-four hour cultures were inoculated into 1% peptone broth plus 1% of the carbohydrate designated. The cultures were incubated at 37 C. for one week, and the P_H value determined by matching the tubes against the Clark and Lubs[6] standards. In table 7 the P_H values alone are given, since gas was not formed in any case.

The table shows that the carbohydrates are either fermented or not; but in either case the reaction is uniform. There are slight differences in some of the mediums, but they are not important enough for classification; they indicate merely functional differences and as such are negligible.

To compress the table:

Carbohydrates Fermented	Not Fermented
Glucose	Starch
Galactose	Dulcitol
Levulose	Adonitol
Sucrose	Dextrin
Lactose	Inulin
Maltose	Arabinose
Salicin	Raffinose
Mannitol	
Sorbitol	
Glycerol	

[30] Quoted by Winslow and Winslow. Supplement to the 34th annual report of local gov't. bd. containing the report of the Med. officer for 1904-1905, p. 387.

[31] Jour. Path. & Bacteriol., 1908, 12, p. 242.

[32] Jour. Infect. Dis., 1913, 12, p. 432.

[33] Winslow, Rothberg and Parsons: Jour. Bacteriol., 1920, 5, p. 145.

The discrepancy in uniformity of fermentation between this study and that of Winslow and others is possibly due to the fact that they included in their survey strains of Staph. epidermidis, ureae, candidus, tetragenus, candicans, aureus and aurianticus, thereby making a survey of many less active organisms than those employed in our study.

PROTEIN METABOLISM

Decomposition of Peptone to Amino Acids.—As a rule, the only accessible figures of amino acid formation of staphylococci occur scattered through bacteriologic literature where the question at hand was primarily a study of the nitrogen metabolism of several species and

TABLE 7

FERMENTATION OF CARBOHYDRATES

	Arabinose	Dextrose	Galactose	Levulose	Sucrose	Lactose	Maltose	Inulin	Dextrin	Adonite	Salicin	Mannitol	Sorbitol	Dulcitol	Glycerol	Starch	Raffinose
A 1	7.4	6.2	5.9	7.0	6.2	6.2	6.2	7.8	7.1	7.6	6.0	6.2	6.0	7.7	6.0	8.0 — 7.8	
A 2	7.3	4.4	4.7	5.0	5.0	5.0	4.8	7.5	7.1	7.6	6.0	4.6	4.7	7.7	6.0	8.0 — 7.7	
A 3	7.2	4.6	4.7	5.0	4.8	5.6	4.8	7.5	7.1	7.6	6.0	5.0	4.7	7.7	6.0	8.0 — 7.6	
A 5	7.2	4.4	4.6	5.0	4.8	5.0	4.8	7.5	7.6	7.6	5.8	5.0	4.7	7.7	6.0	8.0 — 7.8	
F 1	7.0	4.6	4.6	5.0	4.9	5.0	4.8	7.5	7.8	7.6	6.0	5.4	4.7	7.7	6.0	8.0 — 7.7	
H 2	7.3	4.4	4.7	5.0	4.8	5.0	4.8	7.5	7.1	7.6	6.0	5.2	4.7	7.8	6.0	8.0 — 7.7	
P 1	7.3	4.4	4.7	5.0	4.6	5.1	4.6	7.5	7.1	7.6	6.0	5.0	4.7	7.7	6.0	8.0 — 7.7	
P 2	7.2	4.4	6.0	5.2	4.8	5.0	4.8	7.5	7.1	7.5	5.4	5.0	4.7	7.7	6.0	8.0 — 7.8	
P 3	7.3	4.5	4.7	5.2	4.8	5.0	6.0	7.5	7.1	7.6	6.0	4.6	4.7	7.7	5.0	8.0 — 7.8	
P 4	7.2	4.4	4.7	5.0	4.7	5.1	5.4	7.5	7.2	7.6	6.0	4.6	4.7	7.6	5.0	8.0 — 7.8	
P 5	7.3	4.9	5.1	5.2	4.9	5.1	5.6	7.5	7.1	7.6	6.2	5.4	4.7	7.7	6.0	8.0 — 7.5	
S 2	7.3	4.6	4.7	5.2	4.8	5.4	4.8	7.5	7.1	7.6	6.0	4.6	4.7	7.7	6.0	8.0 — 7.7	
T 1	7.3	4.4	4.7	5.0	4.8	5.0	4.8	7.5	7.1	7.8	6.0	5.3	4.7	7.6	5.2	8.0 — 7.7	
T 2	7.3	4.4	4.7	5.0	4.6	5.1	4.8	7.5	7.2	7.6	6.0	5.2	4.7	7.7	6.0	8.0 — 7.7	
T 3	7.3	4.4	4.7	5.0	5.0	5.0	4.2	7.6	7.2	7.6	6.2	4.6	4.7	7.7	5.0	8.0 — 7.7	
T 5	7.2	4.4	4.7	5.0	4.8	5.1	4.8	7.3	7.2	7.6	6.0	4.6	4.7	7.7	6.0	8.0 — 7.7	
T 6	7.3	4.6	4.7	5.2	4.8	5.0	4.8	7.5	7.1	7.6	6.0	4.6	4.7	7.7	6.0	8.0 — 7.7	
T 8	7.3	4.4	4.8	5.0	4.8	5.6	4.8	7.5	7.0	7.4	6.0	4.6	4.7	7.6	5.0	8.0 — 7.6	
T 9	7.3	4.6	4.9	5.0	4.8	5.2	4.8	7.5	7.1	7.4	6.0	5.2	4.7	7.7	6.0	8.0 — 7.9	
X	7.3	4.4	4.7	5.2	5.0	5.0	4.8	7.5	7.1	7.4	5.4	5.4	4.7	7.0	5.0	8.0 — 7.7	
Control	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0 — 7.2	

one or two strains of staphylococci were fortuitously included. Our object here was to give a definite conception of the amino acid digestion of peptone, and incidentally to use such an expedient for a classification, if possible.

Rosenthal and Patai[34] found that the curve of amino acid production by staphylococcus underwent an initial sharp rise within 24 hours, and this was followed by a more gradual rate of increase until the fifth and sixth day. Also virulent organisms produced more amino acid than the avirulent ones. Their determinations were made by the Sørensen method. The work of Berman and Rettger[35] shows that at the end of 1 week 3 strains of aureus reached an

[34] Centralbl. f. Bakteriol., I, O., 1914, 73, p. 406.

[35] Jour. Bacteriol., 1918, 3, p. 367.

amino acid figure equivalent to 47 c c of 20/NaOH, and one strain of albus, a figure of 52 c c of 20/NaOH. They also used the Sørensen method.

Benton[36] recently observed that in 1.5% peptone broth, staphylococcus shows a decrease in amino acidity until the 5th day, with a following rise until the 7th day; in 2% peptone broth, the decrease continues until the 3rd day with a gradual increase until the 9th day; in pure ascitic fluid, after a 1 day decrease, there is a rise until the 4th day. She used the Van Slyke method for amino acid determination.

In our own experiment, a 2% Difco peptone extract broth was employed. The tubes were inoculated with a 24-hour growth and at the end of each day the amino acidity was determined by the Sørensen method (table 10).

TABLE 8

AMINO ACID DECOMPOSITION OF PEPTONE *

	1st Day		2d Day		3d Day		4th Day		5th Day	
	PH	A. A.	PH	A. A.	PH	A. A.	PH	A. A.	PH	A. A.
A 1	7.5	40.0	7.5	48.0	7.5	72.0	7.5	100.0	7.5	44.0
A 2	7.5	60.0	7.7	48.0	7.6	84.0	7.7	112.0	7.9	72.0
A 3	7.5	68.0	7.7	68.0	7.7	88.0	7.7	116.0	7.9	72.0
A 5	7.5	80.0	7.5	72.0	7.6	92.0	7.6	120.0	8.0	76.0
F 1	7.5	68.0	7.5	72.0	7.8	96.0	7.8	120.0	8.0	60.0
H 2	7.5	68.0	7.5	96.0	7.7	112.0	7.8	116.0	8.0	72.0
P 1	7.8	56.0	7.9	56.0	7.8	96.0	7.8	116.0	8.0	72.0
P 2	7.7	56.0	7.7	80.0	7.7	96.0	7.7	116.0	8.0	72.0
P 3	7.5	56.0	7.9	84.0	8.0	96.0	7.7	116.0	7.7	64.0
P 4	7.5	56.0	7.9	72.0	7.9	100.0	7.7	92.0	7.7	68.0
P 5	7.3	52.0	7.4	68.0	7.5	76.0	7.5	76.0	8.0	64.0
S 2	7.7	48.0	7.8	76.0	7.8	72.0	7.8	92.0	8.0	80.0
T 1	7.8	80.0	7.9	116.0	7.9	116.8	8.0	140.0	8.0	68.0
T 2	7.5	56.0	7.9	84.0	7.9	96.0	7.9	80.0	8.0	68.0
T 3	7.5	56.0	7.9	72.0	7.9	96.0	7.9	96.0	8.0	68.0
T 5	7.5	56.0	7.6	72.0	7.8	88.0	7.5	80.0	8.0	40.0
T 6	7.5	56.0	7.8	68.0	7.7	92.0	7.7	72.0	7.9	60 0
T 8	7.7	56.0	7.9	116.0	8.0	116.0	7.7	132.0	7.7	104.0
T 9	7.6	44.0	7.7	80.0	7.8	100.0	7.8	72.0	7.9	64.0
X	7.8	52.0	7.8	72.0	7.8	92.0	7.8	116.0	8.0	64.0
Control	7.0	48.0	7.0	48.0	7.0	48.0	7.0	48.0	7.0	48.0

* In PH column, PH readings are given. In A. A. column, figures represent number of c c of 20/N NaOH required to neutraliz 100 c c of culture.

The significant features brought out are that at the end of the first day there is either a slight increase or decrease in amino acid, then a gradual rise to the 4th day, with a falling off on the 5th day. At the time, we assumed that a maximum had been reached on the 4th day. This checks fairly well with the results of Rosenthal and Patai, but brings our maximum a bit sooner than was the case in Benton's work.

These results differ materially from those obtained with serum broth, but the mediums were of course different. It would seem that in serum broth the amino acids are simultaneously formed and utilized,

[36] Jour. Infect. Dis., 1919, 25, p. 231.

and thus the figures are kept low; whereas in peptone broth, the amino-acidity figures increase rapidly due to the greater amounts of peptone present.

Production of Ammonia.—This test was performed for a double purpose: In the first place, it was interesting to determine what happened to the amino acid formed, and in the second place, to determine whether any differentiation could be made on this basis. The amount of ammonia formed was measured daily for 5 days after incubating at 37 C. The medium employed was composed of 1% peptone and 0.05% K_2HPO_4. The tubes were sealed with paraffin to prevent the escape of ammonia. The determination was made with Nessler reagent, and the cultures were matched against a known standard by

TABLE 9
AMMONIA FORMATION

	2d Day	3d Day	4th Day	5th Day
A 1	6.4	9.72	11.60	21.84
H 2	13.80	19.20	20.40	14.64
H 3	12.00	17.76	20.50	16.44
H 5	12.00	15.36	23.40	16.92
F 1	9.60	20.40	24.00	26.04
H 2	14.16	21.84	27.04	21.00
P 1	12.84	14.60	16.80	16.78
P 2	12.60	14.60	19.20	21.84
P 3	9.60	21.60	30.00	21.84
P 4	17.16	20.40	30.00	32.88
P 5	9.60	14.16	15.48	30.00
S 2	12.96	14.40	28.20	24.00
T 1	24.00	27.00	48.00	48.00
T 2	13.32	30.00	32.88	21.36
T 3	16.78	23.20	24.00	14.64
T 5	14.76	21.60	32.80	28.20
T 6	14.16	17.96	32.80	28.56
T 8	32.80	38.40	64.80	57.12
T 9	14.60	16.68	32.80	14.60
X	19.20	23.40	24.00	16.44
Control	1.98	1.98	1.98	1.98

Figures represent mg. of NH_3 as nitrogen per 100 c c of culture.

means of the Dubosq colorimeter. From table 9 it will be seen (1) that all the cultures produce ammonia, and (2) that amino-acidity and ammonia formation are simultaneous processes. Winslow, Rothberg and Parsons report positive ammonia formation in all but 11 strains out of 180 studied.

Reduction of Nitrates. — Gordon[30] and Winslow,[28] Rothberg and Parsons[33] found that nitrate reduction by staphylococci was a more or less general character. Winslow and Winslow[28] reported only 21% aureus and 13% albus reducers. Kligler's study showed 7 out of 11 aureus and only 1 out of 12 albus reduced nitrates. The more recent work of Winslow, Rothberg and Parsons[33] had the advantage of better technic and should have the greatest weight.

In making our determination, the medium contained 1% peptone, 0.5% K_2HPO_4 and 1% KNO_3. The cultures were incubated for one week at 37 C., and the presence of nitrates was determined by the sulphanilic acid—a—naphthalamine method. All the strains except A were able to reduce nitrates.

Formation of Indol.—In a survey of the literature of indol production by staphylococci, 3 references have been found of a positive nature. Emmerling[38] described the production of indol afer 14 days' cultivation under anaerobic conditions on an egg white medium. Tissier and Martelly[39] reported positive indol by a culture of Staphylococcus albus isolated from meat, and cultivated in a fibrin medium. Distaso[40] isolated an atypical staphylococcus which was an obligate anaerobe and showed inability to attack any sugar, but which was capable of forming indol. The results of the first two are questionable on account of the technic employed, while the third case is concerned with an atypical organism. On the other hand, negative indol production is reported by Buard,[41] Seltzer,[42] Dobrowski,[43] Distaso,[44] Zipfel,[45] Herzfeld and Klinger,[46] Winslow, Rothberg, and Parsons,[33] and Bayne-Jones and Zimmiger.[47]

Our tests were made by cultivating in a medium of 1% peptone and 0.5% K_2HPO_4 at 37 C. Tests for the presence of indol were made on the first, third, fifth, seventh and tenth day after incubation by the para-dimethyl-amido-benzald-benzaldehyde method. All tests were negative.

Action on Milk.—Table 10 gives the reaction of each strain in litmus milk. It will be seen that after 10 days' incubation at 37 C., one strain shows no apparent change in reaction, 11 strains show acid production, and 8 strains show acid with coagulation and liquefaction. The P_H values in lactose broth has been placed alongside the milk reactions. As was expected, the reaction coincides.

Liquefaction of Gelatin.—In determining gelatin liquefaction, an effort was made toward a quantitative study. The technic employed was to inoculate gelatin tubes with 0.1 c c of a 24-hour broth culture (diluted if necessary to insure an even turbidity). The amount of gelatin liquefied was measured by determining the number of c c from a mark drawn at the original level of the gelatin to the level of the

[38] Berlin der Deutsch. Chem. Gessellsch., 1896, 29, p. 2721.
[39] Ann. de l'Inst. Pasteur., 1910, 24, p. 865.
[40] Centralbl. f. Bakteriol., I, O., 1912, 62, p. 433.
[41] Compt. rend. Soc. de biol., 1908, 65, p. 158.
[42] Centralbl. f. Bakteriol., I, O., 1909, 51, p. 465.
[43] Ann. de l'Inst. Pasteur., 1910, 24, p. 595.
[44] Centralbl. f. Bakteriol., I, O., 1911, 59, p. 102
[45] Ibid., 1913, 67, p. 572.
[46] Ibid., 1915, 76, p. 1.
[47] Bull. Johns Hopkins Hosp., 1921, 32, p. 299.

nonliquefied gelatin. The cultures were incubated at 20 C. for 21 days, unless the gelatin was entirely liquefied before that time, when the liquefaction was estimated.

TABLE 10

SHOWING ACTION ON MILK

	1 Day	3 Days	5 Days	7 Days	10 Days	Lactose
A 1	No change	No change	No change	No change	No change	6.2
A 2	Acid	Acid	Acid	Acid	Acid	5.0
A 3	Acid	Coagulation	Coagulation	Liquefaction	.....	5.6
A 5	Acid	Coagulation	Coagulation	Liquefaction	.....	5.0
F 1	Acid	Coagulation	Coagulation	Liquefaction	.....	5.0
H 2	Acid	Coagulation	Coagulation	Liquefaction	.....	5.0
P 1	No change	Acid	Acid	Acid	Acid	5.1
P 2	No change	No change	Acid	Acid	Acid	5.0
P 3	No change	Acid	Acid	Coagulation	Liquefaction	5.0
P 4	Acid	Acid	Acid	Acid	Acid	5.1
P 5	Acid	Acid	Acid	Acid	Acid	5.1
S 2	No change	No change	No change	Acid	Acid	5.4
T 1	No change	Acid	Acid	Coagulation	Liquefaction	5.0
T 2	No change	No change	No change	Acid	Coagulation	5.1
T 3	No change	Acid	Acid	Acid	Acid	5.0
T 5	No change	No change	No change	No change	No change	5.1
T 6	No change	No change	No change	Acid	Acid	5.0
T 8	Acid	Acid	Acid	Acid	Acid	5.6
T 9	Acid	Acid	Acid	Acid	Coagulation	5.2
X	Acid	Coagulation	Liquefaction	Acid	.....	5.0

TABLE 11

LIQUEFACTION OF GELATIN

	Amount Liquefied	No. of Days
A 1	No liquefaction	21
A 2	5.2 c c	19
A 3	No liquefaction	21
A 5	7.1 c c	19
F 1	No liquefaction	21
H 2	No liquefaction	21
P 1	6.0 c c	19
P 2	1.0 c c	21
P 3	5.5 c c	18
P 4	No liquefaction	21
P 5	No liquefaction	21
S 2	3.0 c c	19
T 1	6.7 c c	17
T 2	5.5 c c	17
T 3	0.9 c c	21
T 5	3.8 c c	19
T 6	No liquefaction	21
T 8	No liquefaction	21
T 9	4.1 c c	21
X	2.0 c c	21
Control	No liquefaction	21

Manner of Liquefaction: Some time later the study of gelatin liquefaction was extended by an observation of the manner of liquefaction. Table 12 gives a graphic representation of the findings. Up to the 10th day, let us say, there is a pseudodifferentiation of 2 types: one type giving a saucer-shaped liquefaction and the second type giving

a cone-shaped liquefaction. After that the liquefaction proceeds uniformly in all the cultures by stratification. The difference, however, is so superficial that we would hardly suggest a classification on this characteristic.

One significant feature brought out by the tables is the ability of six cultures to attack gelatin-cultures which did not a year previously manifest this ability. This shows above all the variability of the organisms to be classified—a variability which emphasizes the fact that in order to classify staphylococci we must depend on more substantial characters than functional differences.

TABLE 12

RESULTS OF AGGLUTINATION *

	Serums				
	A 1	A 5	P 1	T 9	L 1
A 1	5120	0	20	40	20
A 2	0	2560	1280	0	1280
A 3	0	2560	1280	0	1280
A 5	0	2560	1280	0	1280
F 1	1280	20	20	40	20
H 2	80	320	320	0	1280
P 1	0	2560	1280	20	1280
P 2	1280	0	0	0	0
P 3	0	2560	640	2560	1280
P 4	80	640	320	0	640
P 5	0	320	640	40	1280
S 2	1280	0	0	0	0
T 1	0	2560	640	0	1280
T 2	640	2560	640	320	1280
T 3	0	2560	1280	0	1280
T 5	640	20	0	0	1280
T 6	0	2560	1280	40	0
T 8	640	0	0	20	40
T 9	640	2560	1280	640	1280
X	1280	2560	640	1280	1280
C 15	2560	160	80	80	20
C 16	640	0	20	20	20
C 18	640	0	20	20	20
J 1	80	1280	1280	80	1280
L 1	0	1280	1280	0	1280

* Figures represent the dilution at which agglutination was observed by naked eye reading. All controls were negative.

Reduction of Methylene Blue.—At the December, 1921, meeting of the Am. Assn. of Bacteriol., Avery reported his investigation of the use of methylene blue in differentiating hemolytic streptococci from human and dairy sources. He found that dairy strains—bovine and cheese—reduced methylene blue, but that the human strains did not. Because of these results, we tried reducing methylene blue by our staphylococcus strains. The technic of the test consisted in adding to a 24-hour broth culture varying dilutions of methylene blue, and covering with sterile paraffin. The cultures were reincubated for a second day,

when the results were read. It was found that all strains reduced or decolorized methylene blue at dilutions of 1 : 50,000 and 1 : 25,000; they showed partial decolorization at dilution of 1 : 10,000, except strains T8, C15 and J1, which were negative; and at a dilution of 1 : 1,000 all the strains were negative.

Hydrolysis of Sodium Hippurate.—Ayers and Rupp [48] found that hemolytic bovine streptococci could be differentiated from the human by the fact that the former could split sodium hippurate into glycocoll and benzoic acid. We employed this test in our study to determine whether such a procedure would be of value in differentiating the staphylococci. The medium employed contained 1% peptone, 1% sodium hippurate 0.015% K_2HPO_4, and the reaction was adjusted to P_H 7.2. The cultures were incubated at 37 C. for 7 days. At that time hydrolysis was determined by adding 0.5 c c of a 7 % $FeCl_3$ solution for every 2 c c of the culture medium; if hydrolysis had taken place an insoluble precipitate was formed, whereas the mixture became clear on standing several minutes if hydrolysis had not taken place. All the cultures were able to split sodium hippurate.

RELATION TO VIRULENCE

Although Neisser and Wechsberg [2] showed that aureus and albus strains alike are capable of hemolytic activity, their experiment seems to indicate that purely saprophytic forms never attain this faculty. This was corroborated later by Kutcher and Konrich [22] and also by Koch.[23] Noguchi in presenting his results stated that hemolysis was proportional to the virulence of a strain, but the evidence he presents does not justify such a conclusion. Montegazza [49] was unable to demonstrate any definite relation between the intensity of an infection and the quantity of hemolysin produced.

In approaching an answer to the question of inter-relationship between virulence and hemolysis, two methods present themselves—either hemolytic strains will prove to be virulent, or nonhemolytic strains will be avirulent.

Following the first method, then, strains A5, P1, P3 and T9, all definitely hemolytic, were used. Twenty-four-hour broth cultures of each were inoculated in 1 c c quantities into the peritoneum of separate mice. No causalties occurring, the mice were killed, the peritoneums were washed with sterile saline, and the washings injected into a fresh mouse. Incidentally, cultures were made of the peritoneal exudate and heart blood as a check. This procedure was carried successively for

[48] Personal communication.
[49] Biochem. Centralbl. 1908, 8, p. 226.

3 days with 3 mice for each strain. After the third mouse, in no case was staphylococcus demonstrable by smear or culture from the peritoneum indicating complete overwhelming of the 4 strains. Cultures of the heart blood, which were made to test the invasive powers of the 4 strains, were negative each day. Here, if anything, the virulence of the strains should have increased by the animal passage, but instead the organisms decreased, the more resistant organisms lasting until the third passage. This would indicate that hemolysis is quite independent of virulence.

Later, in attempting to isolate a virulent strain, 3 different strains from pus were injected into rabbits. Two strains injected intravenously in amounts of 3 c c of a 24-hour broth culture caused no apparent effect. The third strain caused death in 0.5 c c amounts within 2 days, and 0.25 c c amounts within 1 week, presenting in this case typical staphylococcus lesions. This strain was used in our serologic work and designated as L1. The point of interest here, however, is that although the 3 strains were distinctly hemolytic, only 1 proved to be sufficiently virulent to kill a rabbit. The combined evidence of these 7 strains makes plausible the conclusion that hemolytic strains are not necessarily virulent.

The second method—that nonhemolytic strains would prove to be avirulent—was not tried. Nonhemolytic strains were not isolated during the course of the entire investigation. However, a glance at table 4 at this point will show that strains of an undoubtedly saprophytic character are hemolytic. In a general way, perhaps, the strains requiring the greatest time for hemolysis are probably the least virulent of any; but, on the other hand, the strains giving most rapid hemolysis may be saprophytic.

LEUKOCIDIN ACTIVITY

It was not the purpose in this experiment to make a study of the leukocidin produced by staphylococci. The subject has been well worked out. The purpose was rather to determine whether hemolytic activity bears any relation to leukocidin activity.

Van de Velde[50] first demonstrated leukocidin by filtration in 24-hour cultures. Later he and Denys[51] showed that the leukocidin was not specific, but was a metabolic product which destroyed other tissue cells as well as leukocytes. Bail[52] obtained a maximum production of leukocidin in 11 days. Neisser and Wechsberg[2] added considerably to the knowledge of staphylococcus leukocidin. Making use of the reduction of methylene blue by leukocytes, they found that leukocidin appears in filtrates after 4 days and reaches

[50] La Cellule, 1894, 10, p. 403.
[51] Ibid., 1895, 11, p. 395.
[52] Arch. f. Hyg., 1898, 32, p. 133.

a maximum after 1 week; that leukocidin was produced by white and orange strains; that the more virulent the strain the more leukocidin produced; that leukocidin was destroyed by heating at 56 C.; that normal horse and immune serum possesses antileukocidin; that leukocidin does not attack kidney cells.

In making our tests the same strains used for hemolytic activity were used. The cultures were inoculated each day into 10% serum broth for 16 days so that on the 17th day we had 16 cultures of each strain of from 1 day to 16 days old. The cultures were then centrifuged at high speed for 5 minutes, and 1 c c of the supernatant fluid was used for the test.

Leukocytes were obtained by injecting 8-10 c c of sterile aleuronat into the pleural cavity of guinea-pigs, and after 15 hours the animals were bled to death and the pleural exudate removed with a capillary pipet. An equal amount of 1.5% sterile sodium citrate was added to the cells to prevent coagulation.

The presence of leukocidin was determined by the methylene blue reduction test. The methylene blue consisted of 1 c c saturated solution of methylene blue, 20 c c absolute alcohol, and 29 c c distilled water. The minimum quantity of leukocytes to reduce methylene blue was first measured by using different amounts of leukocytes varying from 0.2 c c to 2 c c, the volume being made equal through the series with sterile salt solution. Two drops of methylene blue were added, and then the mixture was covered with a layer of sterile liquid paraffin to prevent reoxidation from the air. The tubes were incubated at 37 C. for 2 hours.

To twice the minimum quantity of the leukocytes found necessary to give reduction of methylene blue was added 1 c c of the supernatant centrifuged culture. The tubes were incubated at 37 C. for 1½ hours, when 2 drops of methylene blue and liquid paraffin were added. Incubation was continued for 2 hours more when the readings were made. In case of reduction, no leukocidins were present, since the leukocytes had not been injured.

It was found that leukocidin appeared on the 4th day and disappeared on the 8th day; and that only strains H2 and T9 produced leukocidins. Thus it is seen that H2, which did not show hemolysin production in broth cultures, produces most leukocidin, and A5, which produced most hemolysins, does not produce leukocidins. A1 is negative in both cases, while T9 is positive in both cases. However, strains A5 and H2 indicate distinctly that hemolytic and leukocidin activity are not dependent on each other.

Theoretically we would expect that the amount of leukocidin produced would bear a relation to the virulence of a strain, for the latter would depend to some extent on the former. Since virulence and hemolysis were found to be individual characters, it was hardly supposed that hemolysis would show any dependence on leukocidin production.

III. SEROLOGIC REACTIONS

As a final analysis, recourse was taken to differentiate the hemolytic staphylococci on a serologic basis. The impression is that although biochemical reactions may vary, serologic reactions if once positive will always remain positive. So, for example, the agglutinability of an organism may fluctuate quantitatively, but not qualitatively. For no other reason, then, this part of the work seemed to have the greatest promise. Both deviation of complement and agglutination tests were made, and the agglutination tests were supplemented by absorption tests.

In preparing immune serums, strains A1, A5, P1, T9 and L1 were employed. Salt suspensions were made from agar slants and rabbits were injected intravenously in 3 day periods, with 2 days between each period. Five-tenths c c of the suspensions was injected the first period, and this was increased 0.5 c c each period until a serum of sufficiently high titer was obtained.

COMPLEMENT FIXATION

The literature on the complement fixation of staphylococci is scant. The one reference available was that of Kolmer, Trist and Yagle [53] in relation to influenza. Using a Staphylococcus aureus antigen, they were unable to get fixation with either normal or influenza serum.

The antigens used in these experiments were suspensions of 24 cultures to which were added 0.1% formaldehyd. The preparation of the serum has already been described.

After going through the preliminaries of obtaining antigenic and complementary doses, the tests were made by incubating at 37 C. It was found that all 5 serums gave fixation with all of the antigens. There appears to be no qualitative differentiation of the different strains.

One more step was taken, and that was to determine whether there might be quantitative separation into groups by complement fixation. Four strains were picked at random, and the serum used in dilutions of 1:50, 1:100, 1:150. The results did not warrant extending the

[53] Jour. Infect. Dis., 1919, 24, p. 583.

work to include all the strains. No sharp difference in the ability of the strains to fix complement was manifested, as the serums were increased in dilution.

It would seem, therefore, that staphylococci are able to fix complement in more or less the same degree. Further, the reaction is a specific one for antigens prepared of streptococci and B. friedländer were unable to prevent hemolysis. But no evidence is given of a possible classification of staphylococci by complement fixation—either in a qualitative or quantitative way.

This is not in the least surprising, however, when we recall that complement fixation does not show divisions into groups with those cocci which have been proved to be of different serologic types by agglutination reactions.

AGGLUTINATIONS

The agglutination reactions of the staphylococci have been studied by several investigators. Kolle and Otto[20] found that immunized serum distinguished the pathogenic from the nonpathogenic forms. This was confirmed by Klopstock and Bockenheimer,[53a] Van Durme,[3] Proscher,[54] Kutscher and Konrich,[22] Veiel,[55] Fraenkel and Baumann[56] and Montegazza.[49] Trincas[57] states that serum prepared with hemolytic strains shows strong agglutination with hemolytic strains, and slight agglutination with nonhemolytic strains; and vice-versa. Walker and Adkinson[58] found that an aureus immune serum would agglutinate aureus and not albus strains; and that an albus immune serum would agglutinate albus and not aureus strains.

Our object was to group staphylococci by agglutination into as many serologic groups as would evidence themselves, without regard to virulence or pigment. The same serums used in the complement-fixation test were used for agglutination, and the same antigens also, except that they were diluted until their turbidity equaled that of the Dreyer standard for the typhoid group agglutinations. The agglutinations were set up in serum dilutions of 1 : 10 and going as far as was necessary to include the agglutination titer of the respective serums. The serum dilutions and antigens were added in 0.5 c c amounts each, and incubation was effected in a water bath at 56 C. for 16 hours.

In table 12 the figures represent the dilution at which final agglutination was observed with naked eye. There was present in the serums a proagglutinoid zone.

An analysis of the table shows that serum A1 agglutinates strains A1, F1, P2, S2, T2, T5, T8, T9, X, C15, C16 and C18. Serums A5,

[53a] Centr. f. Bakt., 1903, 34, p. 437.
[54] Arch. f. klin. Chir., 1903, 72, p. 325.
[55] München. med. Wchnschr., 1904, 51, p. 13.
[56] Ibid., 1905, 52, p. 937.
[57] Biochem. Centralbl., 1908, 8, p. 609.
[58] Jour. Med. Res., 1917, 35, p. 373.

P1, and L1 agglutinate strains A2, A3, A5, H2, P1, P3, P4, P5, T1, T2, T3, T6, T9, X, J1, and L1. Serum T9 agglutinates P3, T2, T9 and X. Serums A5, P1 and L1 are unquestionably the same since they give the same reactions. It will be noted that strains T2, T9 and X are agglutinated by all the serums, and P3 by all the serums except A1. Aside from these atypical agglutinations, the strains fall definitely with one serum. Apparently, then, the agglutination tests give the following grouping:

I.—A1, F1, P2, S2, T5, T8, C15, C16, C18.

II.—A2, A3, A5, H2, P1, P4, P5, T1, T3, T6, J1, L1.

III.—T2, T9 X and possibly P3.

TABLE 13

RESULT OF ABSORPTION TESTS ANTIGENS *

	Serum Absorbed With						
	A 1-A 1	A 1-X	T 9-P 3	T 9-T 2	L 1-A 5	L 1-P 3	L 1-T 9
A 1	0	4800	—	—	—	—	—
A 2	—	—	—	—	0	300	600
A 3	—	—	—	—	0	300	600
A 5	—	—	—	—	0	300	600
F 1	0	+	—	—	—	—	—
H 2	—	—	—	—	600	300	600
P 1	—	—	—	—	0	300	+
P 2	0	+	—	—	—	—	—
P 3	—	—	0	2400	600	0	1200
P 4	—	—	—	—	0	300	+
P 5	—	—	—	—	0	300	+
S 2	0	+	—	—	—	—	—
T 1	—	—	—	—	0	300	+
T 2	—	0	0	0	300	0	0
T 3	—	—	—	—	0	300	+
T 5	0	+	—	—	—	—	—
T 6	—	—	—	—	0	300	+
T 8	0	+	—	—	—	—	—
T 9	0	0	0	0	300	0	0
X	600	0	0	150	600	0	0
C 15	0	+	—	—	—	—	—
C 16	0	+	—	—	—	—	—
C 18	0	+	—	—	—	—	—
J 1	—	—	—	—	0	300	+
L 1	—	—	—	—	0	600	1200

* — indicates that strain did not agglutinate prior to absorption; figures represent dilution of final positive agglutination; + indicates no test. All controls were negative.

ABSORPTION TESTS

In order to further identify the groups suggested by the agglutination reactions absorption tests were conducted, employing the technic of Small and Dickson.[59] One c c of a 1:10 dilution of the immune serum was mixed with 4 c c of the concentrated antigen in a sterile centrifuge tube. This amount of the antigens was found sufficient to

[59] Jour. Infect. Dis., 1920, 26, p. 230.

absorb the homologous agglutinins after 4 hours' incubation at 37 C., the tubes being shaken at half-hour intervals. After this period of incubation the tubes were centrifugalized and the supernatant serum dilution (1:50) was drawn off and agglutinations carried out as described.

Serum A1 was absorbed with strain A1 and X; serum T9 with P3 and T2; serum L1 with A5, P3 and T9, and agglutinations performed against the antigens which agglutinated with the respective serum before absorption. The results are presented in table 13.

The absorption tests confirm the groups found by agglutination. Group 1 remains as was found, but in group 2, H2, is placed in a subgroup because although it agglutinates with the same serums as A5, absorption by A5 does not remove agglutinations for H2. In group 3, P3 is placed in a subgroup. P3 removes agglutinins for all members of group 3, but the other members of group 3 do not remove agglutinins for P3.

Revising our classification, then, we would have:

Group 1	Group 2	Group 3
A 1	A 2	T 2
F 1	A 3	T 9
P 2	A 5	X
S 2	P 1	Subgroup
T 5	P 4	P 3
T 8	P 5	
C 15	T 1	
C 16	T 3	
C 18	T 6	
	J 1	
	L 1	
	Subgroup	
	H 2	

DISCUSSION OF SEROLOGIC REACTIONS

The use of complement fixation in determining types among the staphylococci appears to be worthless. Although staphylococci do fix complement, no grouping appeared possible, either quantitatively or qualitatively. Nor is this surprising—on the contrary, it is more or less what was to be expected. Complement fixation has been disappointing in its inability to differentiate types—probably because the immunity established although specific for the particular species is general and not sufficiently specialized to detect individual types.

Agglutination, however, has already been proved to be an efficacious means of detecting types. Furthermore, agglutination is a fixed quality, and one which is considered reliable. So that, when the statement is

made that virulent types agglutinate only with serums prepared from virulent strains, there must be an error somewhere. The properties of virulence are obviously among the most unstable of bacterial characters. Culture on laboratory mediums renders a virulent strain nonpathogenic in a very short time. Yet it is scarcely conceivable that the immunity reactions are as readily modified. By way of illustration: Strain L1, which was distinctly pathogenic, was used for the preparation of immune serum before it could have undergone avirulence; but its serum did agglutinate other strains, including A5, P3 and T9, all 3 of which were proved nonpathogenic. It may be that A5, P3 and T9 were pathogenic at some time or another, but at the time the test was made they were not pathogenic. It seems clear to us that virulence does not dictate the group into which a staphylococcus shall fall.

Nor does it seem plausible that hemolytic activity is the basis of agglutination grouping. We have been unable to obtain absolutely non-hemolytic cultures, and have been unable to establish this point conclusively. However, we were able to get these groups among hemolytic organisms, whereas if hemolysis were the fundamental of the grouping, we should have obtained agglutination of all our strains by all our serums.

Regarding the association of pigment and agglutination, this much can be said: Occasionally, there may develop on a plate streaked with a pure culture, colonies varying appreciably in intensity of pigment, from which, as Sullivan [60] has shown, quite distinct types may be derived by selection of the extremes. Yet it does not seem probable that the parent strain in such a case would vary from its successor in its agglutination reactions. More relevant, however, strain J1, which is an albus, did agglutinate with serums A5, P1 and L1, which were prepared from antigens of varying shades of orange. An analysis of the pigment and agglutination tables, with this one exception cited, bears out the contention of Walker and Adkinson [58] in a general way. The members of group 1 are of a light pigment—either white or of a light shade of yellow, which without the refined technic of Winslow and Winslow [28] would easily be called a white.

A study of the tables of the different biochemical reactions shows no definite relationship between the agglutination groups and these reactions. In a very general way Group 1 seems to contain the less active strains, but it also contains some rather active strains. Groups 2 and 3 possess none of the light pigmented nor any of the less active strains.

[60] Jour. Med. Res., 1905, 14, p. 209.

These immunologic groups will perhaps explain the variations experienced in curative and prophylactic inoculations of either the organisms or serum. Stock vaccines, for example, will not necessarily be specific, nor will immune serum prove to be efficacious unless it falls into the same group. But having determined the group or type of staphylococcus under question, we can employ specific material either prophylactically or curatively.

CONCLUSIONS

Staphylococci produce a hemolytic substance in broth which appears on the 6th day, reaches a maximum at the 9th or 10th day and then disappears between the 13th and 16th day.

This hemolytic substance is thermolabile, is unaffected by the presence of carbohydrates and appears to be associated with proteolysis and possibly autolysis.

All cultures of staphylococci isolated during the course of this investigation appear to be hemolytic—only the time of its manifestation is in some cases considerably delayed.

Hemolytic cultures did not lose their hemolytic powers by continued transplantations into blood-free mediums for a period of more than four months.

Hemolytic activity shows no relationship to any of the biochemical reactions studied.

Staphylococci fix complement specifically, but cannot be classified by such an expedient.

Three groups seen definable of the 25 strains studied by agglutinations and absorption test, with 2 ill-defined subgroups—one each under group 4 and group 3.

These groups apparently bear no relationship to virulent hemolysis or biochemical activity. Group 1 appears to include the light pigmented and less active strains.

These groups may account for the variations experienced in the past in the use of serum and vaccines.

The writer wishes to express his sincere gratitude to Doctors A. C. Abbott and D. H. Bergey for their invaluable assistance in advice, criticism and encouragement.